MORPHOLOGICAL COLOR CHANGE IN THE FIDDLER CRAB, UCA PUGNAX (S. I. SMITH)[1,2]

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Morphological color changes have been defined (Green, 1963) as enduring modifications of the pigmentary system resulting from production or destruction of pigment and/or chromatophores. Morphological color changes can occur in a variety of ways: (1) by an increase (decrease) in the number of chromatophores per unit area of body surface (Bowman, 1942; Green, 1963, 1964); (2) by an increase (decrease) in the pigment concentration (Brown, 1934; Green, 1963); or (3) by a combination of the above.

Babak (1912) postulated a relationship between physiological and morphological color changes based on the state of dispersion of pigment within the chromatophores. This relationship, subsequently called Babak's Law, stated that the maintenance of a pigment in a concentrated state within the chromatophore was correlated with a reduction in the quantity of that pigment. Conversely, pigment dispersion was associated with pigment production.

The present study deals with part of the pigmentary system of the fiddler crab, *Uca pugnax,* and with its capacity to undergo morphological color changes.

Materials and Methods

Crabs were collected on the salt flats at Sippewissett Beach, Cape Cod, Massachusetts. The animals were brought to the laboratory and placed in a large tank with continuously running sea water covering the bottom to a depth of $\frac{1}{2}$ inch. Crabs were selected at random from this group (usually about 100 animals) for the various experiments. In all cases males were used and the great chela was removed by forcing the animal to autotomize the appendage. This procedure reduced the hemocoel space and thereby increased the efficiency of incorporation of radioactive precursors.

Tyrosine-C^{14} was injected into the crabs as a precursor of the black pigment. In one series of experiments dihydroxyphenylalanine-C^{14} (DOPA) was used in place of tyrosine. All injections were made into the hemocoel through the arthrodial membrane between the coxopodite and the ischiopodite of the fourth walking

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leg. After the injection of the tyrosine the crabs were placed on various backgrounds. The animals were unfed during the experimental period (which amounted to at most 20 days for any one animal). After the appropriate time on the background the approximate area of the carapace was determined. The carapace was then dissected from the animal and freed of adhering tissues. This preparation was composed of the exoskeleton lined with epidermis in which the pigment cells lie. The carapace was cut into four pieces and dropped into a stainless steel centrifuge tube. Ten ml. of distilled water were added. The material was sonicated in a M. S. E. sonicator with a $\frac{3}{4}$-inch probe for 2.5 minutes at 20 kc. An additional 10 ml. of water were added after the suspension was decanted and the process was repeated. All of the pigment granules were removed from the hypodermis after 2.5 minutes of sonication; the additional period of sonication served as a rinse. The resulting 20 ml. of dark colored fluid were centrifuged (2100 g) in an International centrifuge for 20 minutes. A black pellet was found in the tubes after centrifugation. Subsequent re-suspension and re-centrifugation with 5 ml. of distilled water, for a total of three washings (in all), was sufficient to remove any extraneous material. The pigment was then suspended in a minimum quantity of water (less than 1 ml.) and pipetted onto aluminum planchets ringed with wax. The suspension was dried over a hot plate and the radioactivity of the pigment measured with a thin-window Geiger tube.

In the following discussion the values reported are the mean number of counts per minute per square centimeter of carapace $\pm$ the standard error of the mean. In all cases the count has been corrected for background radiation. The samples were not corrected for self-adsorption which may be significant; therefore these measurements represent relative and minimal values for incorporation.

Results

Identification of the pigment

The fiddler crab, *Uca pugnax,* has black, red, white and yellow chromatophores. The black and white chromatophores play the major role in determining the shade of the animal. The dark pigment within the black chromatophores has not been previously characterized. In the present work it is identified partially on the basis of its solubility and reaction to strong oxidizing agents. The black pigment was treated with 1 N HCl, 95% and 100% ethyl alcohol, 95% and 100% methyl alcohol, acidic ethyl and methyl alcohols (HCl 5% v/v), acetone, ethylene chlorhydrin, and 0.5 N NaOH. The pigment was soluble only in the last two of the listed solvents. The extracted and washed pigment granules were bleached by 10% hydrogen peroxide in 24 hours at 5° C.

Incorporation studies

Injected tyrosine-C^{14} or DOPA-C^{14} was incorporated into the black pigment *in vivo.* The radioactive label was not removed by exhaustive washing of the extracted pigment with distilled water, 95% or 100% ethyl alcohol. Precipitation of L-tyrosine-1-C^{14}-labeled pigment from 0.5 N NaOH solutions with 0.1 N HCl yielded precipitates with levels of radioactivity not different from that of the untreated pigment.

As a further check on the incorporation of C^{14} into the black pigment, crabs were injected with 0.5 μC. of L-valine-1-C^{14} and with 0.5 μC. of glycine-1-C^{14}. After 24 hours on neutral background with natural lighting no significant levels of radioactivity could be found in the pigment extracted from crabs injected with either amino acid.

Concentration curve

Forty crabs, in groups of four, received varying amounts of DL-tyrosine-2-C^{14}. After 24 hours on a neutral background with natural lighting, the area of the carapace was determined and the pigment extracted from the epidermis. Interpo-

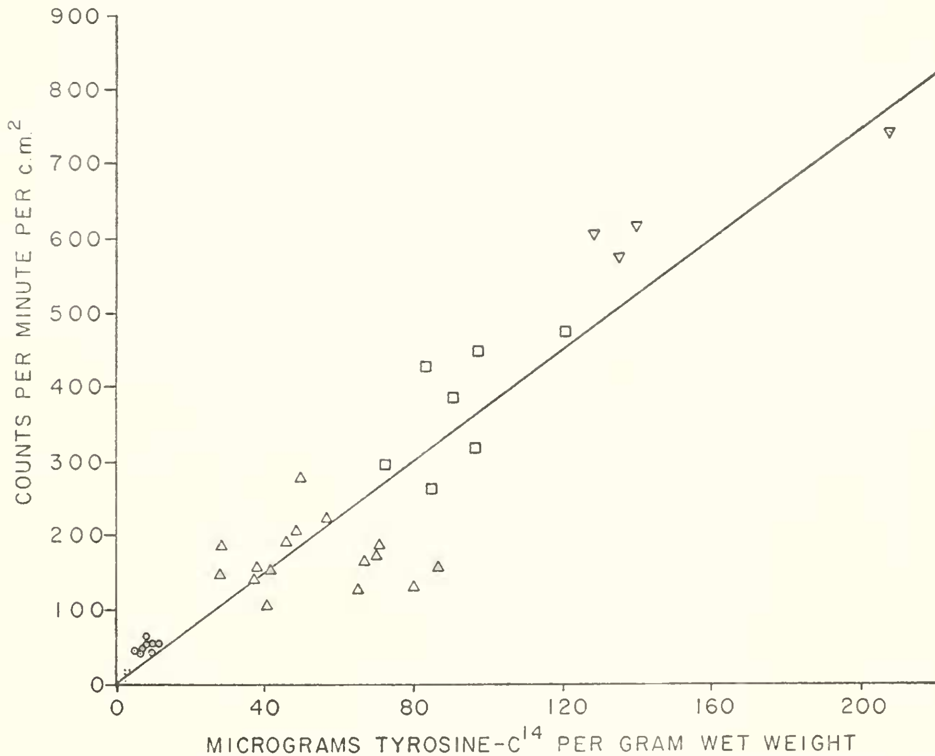

FIGURE 1. The relationship between the counts per minute per square centimeter of carapace of the extracted pigment and micrograms DL-tyrosine-2-C^{14} injected per gram wet weight. The linear regression line has been calculated and the $P = 0.95$ confidence interval of the slope is given. $Y = 3.72X + 5.35$. $3.27 \leq b \leq 4.17$. $P < 0.001$. Solid dots = 0.01 μC.; ⊙ = 0.10 μC.; △ = 0.50 μC.; ⊡ = 1.00 μC.; ▽ = 2.00 μC.: microcuries injected per animal.

lation from a standard curve relating the area of the carapace to the wet weight of dechelate male crabs gave the total body weight. On this basis the μg. of tyrosine-C^{14} injected per gram wet weight of the crabs was calculated. A regression analysis of μg. tyrosine injected per gram wet weight against counts per minute per square centimeter of carapace of extracted pigment was undertaken.

The relationship between $\mu g.$ tyrosine injected per gram wet weight and the radioactivity of the extracted pigment is linear within the experimental range, 0.88–208.00 $\mu g.$ per gram (0.01–2.00 $\mu C.$ tyrosine-C^{14} injected per animal). (Fig. 1). From this series of experiments it was determined that 1.05% of the injected tyrosine-C^{14} was recovered as labeled pigment.

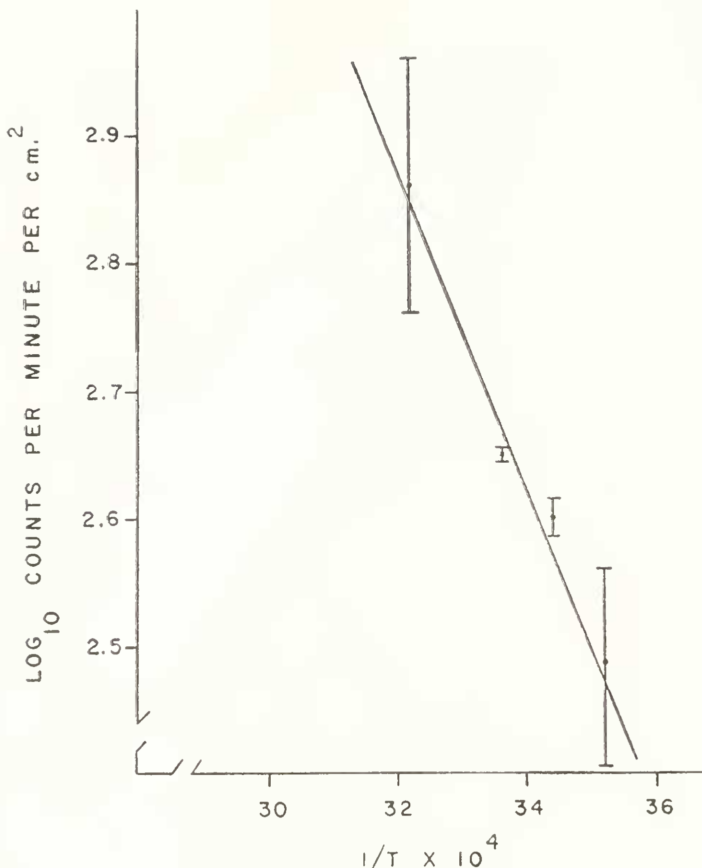

FIGURE 2. Arrhenius plot of data on incorporation of DL-tyrosine-2-C^{14} into the black pigment of the fiddler crab. The linear regression line has been calculated and the $P = 0.95$ confidence interval of the slope is given. $Y = -1.257X + 6.90$. $-0.657 \leq b \leq -1.857$. $P < 0.001$.

Temperature curve

Crabs were maintained at various temperatures for 24 hours after the injection of 1.0 $\mu C.$ per crab of DL-tyrosine-2-C^{14}. The pigment was extracted and the counts per minute per cm.^{2} of carapace were determined. The regression analysis indicated that a significant linear relationship existed between the logarithm of the counts per minute per cm.^{2} of carapace and the reciprocal of the absolute temperature (Fig. 2). The Q_{10} (11–37° C.) is 1.42. From the slope of the Arrhenius

plot of the data, the activation energy (μ) for the incorporation of tyrosine-C^{14} into the black pigment has been calculated to be 5757 calories.

DL-3(3,4-dihydroxyphenyl)alanine-2-C^{14} (DOPA) as a precursor of the black pigment

Fourteen crabs received 0.5 μC. DOPA-C^{14}. The animals were maintained under conditions of neutral background and constant illumination for a period of 24 hours. After this time the animals were weighed and the pigment was extracted from the epidermis. A mean value of 111.9 ± 11.6 counts per minute per $cm.^2$ was obtained. One per cent of the injected DOPA-C^{14} was recovered as labeled pigment.

Incorporation studies with normal animals

Pigment dispersion within the chromatophores of fiddler crabs fluctuates periodically, both in the normal environment and under constant conditions of illumination and temperature in the laboratory (Megusar, 1912; Abramowitz, 1937;

TABLE I

Comparison between radioactivity levels of pigment extracted from crabs injected with tyrosine-C^{14} at noon and at midnight

Treatment	N	Mean ± S. E.	Mean chromatophore stage				
			Hour				
			0	1	2	3	4
Injected at 1200 hours, extracted at 1600 hours	4*	15.70 ± 1.44	5.0	4.8	5.0	4.6	4.4
Injected at 0000 hours, extracted at 0400 hours	4*	15.52 ± 0.42	1.2	1.2	1.0	1.0	1.0
Injected at 1200 hours, extracted at 1600 hours	8**	255.75 ± 30.49	5.0	4.8	4.3	4.3	3.9
Injected at 0000 hours, extracted at 0400 hours	8**	296.75 ± 53.89	1.6	2.5	1.6	1.3	1.2

* 0.1 μC. L-tyrosine-1-C^{14} per animal.
** 1.0 μC. DL-tyrosine-2-C^{14} per animal.

Brown and Webb, 1948; Stephens, 1957). The migration of pigment into preestablished chromatophore channels is controlled primarily by various hormones or chromatophorotropins. Some of these chromatophorotropins appear to be released in a cyclic manner so that the black chromatophore pigment is expanded during the daylight hours and contracted at night.

In an attempt to correlate synthesis of the black pigment with the state of pigment dispersion within the chromatophore (Babak's hypothesis) the following experiments were designed. Crabs were injected with L-tyrosine-1-C^{14} at noon and at midnight. Four hours later the pigment was extracted. The experiment was repeated with injections of DL-tyrosine-2-C^{14}.

The heavy exoskeleton covering the epidermis of the carapace prevented detailed observation of the underlying chromatophores. Therefore, the state of dis-

persion of the pigment in pereiopod chromatophores was utilized as a general index of chromatophore activity. It is possible that the pigment of the carapace chromatophores responds differently than does the pigment of pereiopod chromatophores. No information is available concerning the exact relation between the chromatophore pigments of these areas.

During the four-hour experimental period black chromatophores were staged according to the scheme of Hogben and Slome (1931). From 0000-0400 hours the pigment within the black chromatophores is contracted (stage 1) and during the period 1200–1600 hours it is dispersed (stage 5). Subsequent extraction of the pigment and measurement of its radioactivity levels revealed that there was no difference between the animals injected at midnight and those injected at noon (Table I).

Table II

Comparison between radioactivity levels of pigment extracted from normal and destalked crabs receiving eye-stalk extracts

Treatment	N	Mean ± S. E.	Chromatophore stage
Normal animals			
All animals received 0.5 μC. L-tyrosine-1-C^{14} every four hours for a total of 24 hours			
1. Plus 0.05 ml. of sea water every four hours for a total of 24 hours	4	239.25 ± 10.55	Varies from ~5 at 1200 hours to ~1 at 0000 hours
2. Plus 0.05 ml. eye-stalk extract containing the equivalent of ½ eye-stalk, every four hours for a total of 24 hours	4	262.50 ± 23.30	~5
Destalked animals			
All animals received 0.5 μC. L-tyrosine-1-C^{14} every four hours for a total of 24 hours			
1. Plus 0.05 ml. sea water every four hours for a total of 24 hours	4	381.50 ± 27.75	~1
2. Plus 0.05 ml. eye-stalk extract containing the equivalent of ½ eye-stalk, every 4 hours for a total of 24 hours	4	339.25 ± 25.10	~5

Incorporation studies with eye-stalkless animals (destalked animals)

In the following experiments the effect of an injection of eye-stalk extract on the synthesis of the black pigment was studied. Sixteen crabs were selected, eight normal crabs and eight destalked crabs. The pigment of the black chromatophores of destalked crabs assumes and maintains the contracted position (stage 1), mimicking the condition normally seen only at night (Megusar, 1912; Carlson, 1935). Each crab received 3 μC. of L-tyrosine-1-C^{14} in 0.5-μC. injections every four hours for 24 hours. One-half the animals, four normal and four destalked, also received 0.05 ml. of an eye-stalk extract containing the equivalent of one-half eye-stalk. The eye-stalk extract was made by grinding eye-stalks with sterile sand and artifi-

cial sea water in a mortar. The resulting brei was centrifuged and subsequently heated in a boiling-water bath for five minutes. The material was re-centrifuged and the supernatant was brought to such a volume that 0.05 ml. contained the equivalent of one-half eye-stalk. This technique for the extraction of chromatophorotropin is a variant of that used by Brown (1940). Injection of the equivalent of one-half eye-stalk led to an expansion of the pigment within the black chromatophores (see, also, Sandeen, 1950; Fingerman *et al.*, 1964) and this effect persisted for approximately four hours. Therefore, with the injection of one-half eye-stalk equivalents every four hours the black chromatophore pigment was induced to remain expanded for the entire experimental period of 24 hours. The results of these experiments indicated that the injection of eye-stalk extract had no significant effect on the level of radioactivity of the extracted pigment from either normal or destalked crabs (Table II). However, there was a difference in the level of radioactivity of the extracted pigment between normal and destalked crabs. The level of radioactivity of the destalked crabs' pigment was significantly higher than that of the normal crabs.

TABLE III

Comparison between radioactivity levels of pigment extracted from destalked crabs receiving injections of sea water, eye-stalk extract and/or phenylthiourea

Treatment	N	Mean ± S. E.	Chromatophore stage					
			Minutes after injection					
			0	15	30	45	75	135
All animals received 0.1 μC. L-tyrosine-1-C^{14} plus:								
1. 0.1 ml. Sea Water	4	93.62 ± 4.7	1.4	1.4	1.8	1.2	1.2	1.2
2. ½ eye-stalk	4	97.42 ± 4.5	1.2	4.0	4.0	4.4	4.4	4.4
3. 1 mg. PTU	4	31.50 ± 7.8	1.4	1.8	1.8	2.2	1.2	1.2
4. ½ eye-stalk + 1 mg. PTU	4	24.60 ± 5.3	1.2	3.6	4.2	4.4	4.8	4.4

Inhibition studies

The role of chromatophorotropins and the effect of phenylthiourea (PTU) on the formation of the black pigment were investigated. PTU inhibits the tyrosine-tyrosinase reaction by binding the copper prosthetic group of tyrosinase (Lerner and Fitzpatrick, 1950). The direct action of PTU on the black chromatophores was tested as follows: 15 normal crabs were injected with 1 mg. PTU in sea water (0.1 ml.) each at 1200 hours, 15 control crabs received 0.1 ml. sea water at the same time. The black chromatophores were staged every 15 minutes for one hour and hourly thereafter for a total of four hours. There was no difference in chromatophore stage between the two groups. With this assurance that PTU had no effect on chromatophore stage, 16 destalked crabs received 0.1 μC. of L-tyrosine-1-C^{14} each. These crabs were also treated with eye-stalk extract and/or PTU. After four hours the pigment was extracted and the radioactivity measured (Table III).

Another experiment was performed using eight normal and eight destalked crabs with injections of PTU every six hours for a total of 24 hours. The animals were maintained on a neutral background with natural lighting (Table IV).

From the preceding results it appears that a mechanism involved in the control of pigment dispersion cannot be concerned with pigment elaboration *per se*.

TABLE IV

Comparison between radioactivity levels of pigment extracted from normal and destalked crabs receiving injections of sea water or phenylthiourea

Treatment	N	Mean ± S. E.	Chromatophore stage
All animals received 1.0 μC. of DL-tyrosine-2-C^{14} at the start of the experiment			
Normal animals			
1. 0.05 ml. sea water every 6 hours	4	313.50 ± 25.6	Varies from ~5 at 1200 hours to ~1 at 0000 hours
2. 1 mg. PTU every 6 hours	4	170.25 ± 11.0	Varies from ~5 at 1200 hours to ~1 at 0000 hours
Destalked animals			
3. 0.05 ml. sea water every 6 hours	4	495.50 ± 29.6	~1
4. 1 mg. PTU every 6 hours	4	173.25 ± 17.9	~1

Long-term studies

Twenty crabs each received 0.5 μC. of L-tyrosine-1-C^{14}. The crabs were maintained in glass jars with a small amount of sea water which was changed daily. The jars were kept on a neutral background with natural lighting. One, two, four, eight, and sixteen days after injection, pigment was extracted from members of this group and the radioactivity measured. In a parallel experiment, destalked crabs were similarly treated, although data were obtained only through eight days (Fig. 3). Values for the level of radioactivity of the extracted pigment from destalked crabs were 72% higher than those from normal animals. However, there is no significant change in the level of radioactivity over an 8-day period (destalked crabs) or over a 16-day period (normal crabs) compared to that which obtained after one day.

Background effects

1. Pigment formation

The effect of background shade on pigment formation was investigated. Eight crabs each received 0.5 μC. of L-tyrosine-1-C^{14}. They were placed individually in fingerbowls lined with either white or black cloth moistened with sea water. The crabs were kept in these bowls under conditions of constant illumination. After four hours the crabs were removed, the area of the carapace measured, the pigment extracted, and the radioactivity determined. No significant difference was found in the level of radioactivity of the pigment extracted from crabs maintained on either of the two backgrounds. There was also no significant difference between the black or the white groups and the control animals from Figure 1 (0.5 μC. group).

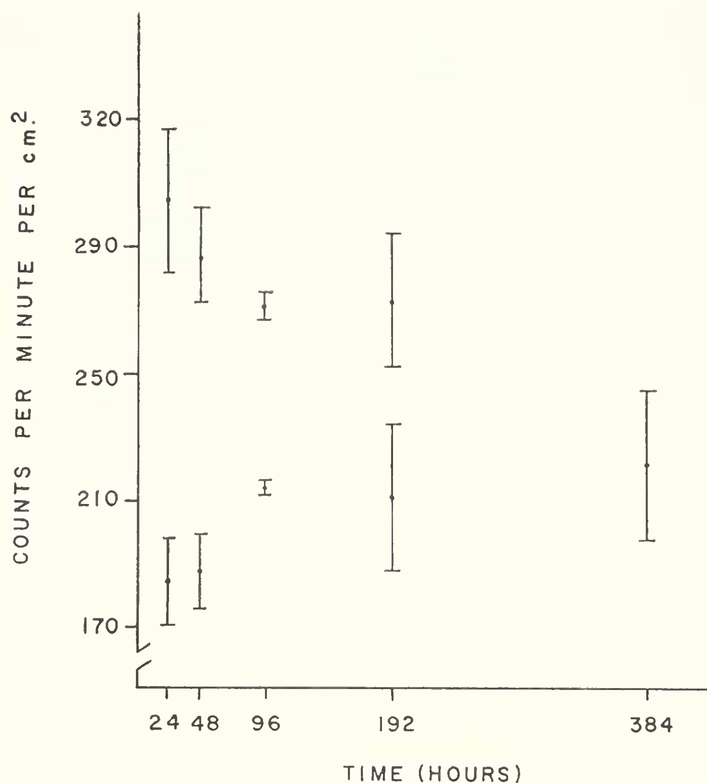

FIGURE 3. The relationship between the counts per minute per square centimeter of carapace of the extracted pigment and length of time spent on neutral background (natural illumination). Upper points for destalked animals, lower points for normal animals. The $P = 0.95$ confidence interval of the slope of the regression line is given. Destalked animals. $-0.50 \leq b \leq 0.02$. Normal animals. $-0.02 \leq b \leq 0.22$.

The effect of background shade on the maintenance of a level of radioactivity in pre-labeled pigment was investigated. Fifteen crabs received 0.1 μC. of L-tyrosine-1-C^{14} each. The crabs were segregated in groups of five and kept in glass jars on a neutral background with natural lighting. Twenty-four hours later four crabs

TABLE V

Comparisons between radioactivity levels of pigment extracted from pre-labeled crabs maintained for 72 hours on white or black backgrounds

Treatment	N	Mean ± S. E.
All animals received 0.1 μC. L-tyrosine-1-C^{14}. Maintained for 24 hours on a neutral background		
Control—24 hours	4	22.2 ± 1.8
Black background—constant illumination—72 hours	4	21.2 ± 3.2
White background—constant illumination—72 hours	4	1.3 ± 0.5

were randomly selected and the pigment extracted. The remaining two groups of five animals were placed on either a white or a black background under conditions of constant illumination. Seventy-two hours later the animals were removed and the radioactivity of the pigment measured (Table V). No significant differences exist between animals maintained on a black background for 72 hours and the control animals (sacrificed after 24 hours on a neutral background). Highly significant differences exist between control animals and those maintained on a white background, and between animals on a white background and on a black background.

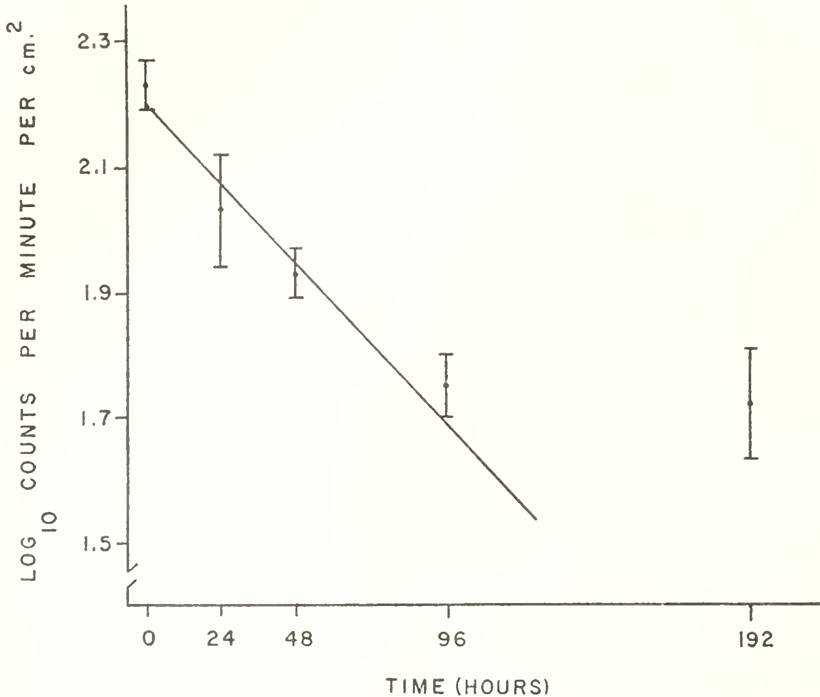

FIGURE 4. The relationship between the logarithm$_{10}$ of the counts per minute per square centimeter of carapace of the extracted pigment and length of time spent on a white brackground (constant illumination). A linear regression line has been calculated for the first 96 hours spent on a white background. The $P = 0.95$ confidence interval of the slope is given. $Y = -0.0053X + 2.208$. $-0.0066 \leq b \leq -0.0039$. $P < 0.001$.

Sixteen crabs received 0.5 μC. L-tyrosine-1-C^{14} each and were placed in groups of four on a neutral background with natural lighting. Twenty-four hours later the crabs were placed on a white background with constant illumination. Twenty-four, 48, 96, and 192 hours later four crabs were removed and the pigment extracted (Fig. 4). The 0.5-μC. animals from Figure 1 served as a control group.

A regression analysis has been carried out, using the logarithm of the counts per minute per cm.^{2} of carapace *versus* length of time on white background (Fig. 4). The greatest decrease (to a level 63.3% of the original level) occurred within

24 hours of placing the animals on a white background. The destruction appeared to occur exponentially for at least the first 96 hours. Fifty per cent of the label was lost from the pigment within 48 hours of placing the crabs on a white background. In a comparable experiment when pre-labeled animals remained on the white or black background for only four hours, no significant differences appeared between these four-hour animals and control animals.

Five crabs were injected with 0.5 μC. of DL-tyrosine-2-C^{14} and placed on a neutral background for 24 hours. They were then transferred to a box which illuminated the crabs from below while presenting a black background on all sides and from above. After 48 hours the crabs were removed and the pigment extracted. The level of radioactivity of the pigment extracted from these crabs was not significantly different from that which was obtained from animals maintained on a neutral background for 24 hours.

Discussion

Nature of the Uca black pigment

The black chromatophores of the fiddler crab, *Uca pugnax,* contain a granular pigment which is easily extracted. This pigment has been called a melanin (witness the name melanophore given to these chromatophores), although definitive proof for the nature of the pigment has never been given. In the present work the *Uca* black chromatophore pigment has been identified as a melanin on the basis of (1) solubility and bleaching reactions; (2) incorporation of tyrosine and DOPA into the pigment; and (3) inhibition of this incorporation by phenylthiourea, a known inhibitor of melanin formation.

Melanin pigments occur as intracellular granules which are relatively insoluble, capable of reducing silver nitrate, and are bleached by strong oxidizing agents (Lison, 1936; Pearse, 1961; Seiji *et al.*, 1963). Ommochrome pigments occur in the integument and eyes of arthropods and have often been confused with the melanins (Needham and Brunet, 1957; Forrest, 1959). Ommochromes can be distinguished from melanins on the basis of their solubility in acidic ethanol (HCl 5% v/v) and in acidic methanol (HCl 5% v/v) (Needham and Brunet, 1957). Because of the solubility properties of the *Uca* integumental black pigment it is highly improbable that this pigment is an ommochrome. The *Uca* black pigment is soluble in 0.5 N NaOH and in ethylene chlorhydrin, as are other melanins (Gortner, 1910; Lea, 1945).

According to the classical scheme for melanin formation in vertebrates, the amino acid tyrosine is enzymatically oxidized to 3,4-dihydroxyphenyl-L-alanine (DOPA). With the help of the same enzyme (tyrosinase) DOPA is oxidized to DOPA-quinone. Subsequently two other quinones are formed which may react with available proteins. The quinone-protein complex then polymerizes to form a black melanoprotein (Mason, 1948; Lerner and Case, 1959; Seiji *et al.*, 1963). In the present work, the incorporation of tyrosine-C^{14} and DOPA-C^{14} into the *Uca* black pigment further indicates that this pigment is a melanin.

Inhibition of the tyrosine-tyrosinase and DOPA-tyrosinase reactions usually occurs by the elimination of copper ions which are necessary for tyrosinase activity (Lerner and Fitzpatrick, 1950; Attie and Khafif, 1964). The majority of known inhibitor substances contain thiourea groups (Attie and Khafif, 1964). In the

present study, phenylthiourea was found to inhibit strongly the incorporation of tyrosine-C^{14} into the melanin. This provides additional evidence for the melanin nature of the *Uca* black pigment.

Characteristics of the incorporation of tyrosine into Uca melanin

The concentration curve shown in Figure 1 indicates that incorporation of tyrosine-C^{14} into the melanin is linearly related to micrograms tyrosine injected. The time period in this experiment was 24 hours; however, comparable levels of radioactivity were found in pigment extracted from crabs which had been injected only four hours before extraction. Therefore, it appears that melanin synthesis occurs rapidly under normal conditions and may be related to the influx of free tyrosine.

The incorporation of tyrosine-C^{14} into the melanin is temperature-dependent, as can be seen from Figure 2. The energy of activation (μ), as calculated from the Arrhenius plot of the data, is 5757 calories. This figure is comparable to values in the literature for the *in vitro* reaction; Gould (1939) has reported a value for μ of 2700 calories for mealworm tyrosinase with catechol as a substrate. Lerner *et al.* (1949) report a Q_{10} (27–37° C.) for mammalian tyrosinase of 1.2, which is equivalent to $\mu = 3577$ calories, with either tyrosine or DOPA as a substrate.

In an attempt to follow the time course of the disappearance of the labeled melanin, crabs were injected with tyrosine-C^{14} and random samples were drawn from the population two, four, eight, and sixteen days after injection. The animals were kept on a neutral background with natural lighting. The level of radioactivity of the extracted pigment did not vary over the experimental period, indicating the stability of the melanin under these conditions.

Eye-stalkless crabs under similar conditions have a level of incorporation approximately 70% higher than do normal animals. They also retain the same level of radioactivity of the extracted pigment over the experimental period (8 days). The metabolism of destalked *versus* normal crustaceans has been investigated by Edwards (1952), Schwabe *et al.* (1952), Kincaid and Scheer (1952), Bliss (1953), Neiland and Scheer (1953), Guyselman (1953), and Carlisle and Knowles (1959). Two of the above papers offer a suggestion as to why the level of radioactivity of extracted pigment should be higher in destalked crabs. Schwabe *et al.* (1952) observed that a hormonal principle from the eye-stalk of *Panulirus japonicus* restrains the growth of the epidermal layer of the integument and with the removal of the eye-stalks, growth of the epidermis occurs. Neiland and Scheer (1953) extended these observations to *Hemigrapsus nudus* and found that normal catabolism of proteins is greatly increased by eye-stalk removal. However, anabolic processes involved in the molt, and especially in the formation of epidermal tissue, are accelerated. In the light of these findings, ablation of the eye-stalks of *Uca* probably leads to increased activity in the epidermis which is reflected by the incorporation of higher levels of tyrosine into melanin.

The responses of the melanophores of the fiddler crab to varying conditions of background shade, illumination, and state of pigment dispersion have been investigated by measuring the incorporation of a radioactive precursor into the pigment. Experiments were performed on crabs whose melanin pigment was in various stages of dispersion, both naturally-occurring and artificially-induced. The normal diurnal cycle of alternate pigment expansion (1200 hours) and contrac-

tion (0000 hours) offered the possibility of testing animals during normally-occurring states of pigment dispersion.

In another experiment injections of eye-stalk extracts every four hours kept the pigment expanded even during the time (0000 hours) when it would normally be contracted. Destalked animals in which the pigment was maximally contracted were also utilized. Injection of eye-stalk extract served to expand the pigment of destalked animals, and repetitive injections maintained it in the expanded state for 24 hours.

The level of incorporation of tyrosine-C^{14} into the melanin of destalked crabs was in all cases considerably higher than that in normal crabs. If this increase is neglected and an attempt is made to correlate the formation of melanin with the degree of pigment dispersion within the melanophore, it is evident that synthesis of melanin is occurring independently of the state of pigment expansion or contraction, whether naturally occurring or artificially induced.

Correlation of sojourn on black or white background with the incorporation of tyrosine into melanin indicated that a four-hour incubation period on either background, under conditions of constant illumination, yielded melanin with similar levels of radioactivity. Furthermore, this level was not different from that found in crabs maintained for 24 hours under conditions of natural illumination. Crabs maintained in total darkness showed a level of incorporation higher than the corresponding animals under normal lighting conditions. However, this result should be re-investigated because of the small number of crabs forming this sample.

Inhibition of tyrosine incorporation

Phenylthiourea has been used successfully as an *in vivo* inhibitor of melanin formation in rats (Dieke, 1947), in ascidian embryos (Whittaker, 1960), in squid embryos and freshly-hatched squids (Arnold, personal communication; Fitzpatrick *et al.*, 1961). The effect of PTU is reversible since pigmentation resumes after withdrawal of the compound (Fitzpatrick *et al.*, 1958; Arnold, personal communication). In the fiddler crab, PTU has no demonstrable effect on the state of pigment dispersion within the chromatophores; therefore, it must be acting as an inhibitor of the tyrosine-tyrosinase reaction rather than having some secondary effect on the melanophore. The radioactivity of the melanin extracted from destalked crabs which received 0.5 mg. PTU per gram wet weight was reduced to a value one-third of that of the non-inhibited destalked animals. In normal crabs inhibited with PTU a 50% reduction in melanin label occurred. In an experiment comparing normal and destalked crabs and their responses to PTU, the level of radioactivity of the melanin extracted from destalked crabs treated with PTU was the same as the level of radioacivity of the melanin extracted from normal crabs treated with PTU. Further analysis would be necessary to decide whether or not this correspondence between levels of radioactivity of pigment in destalked and normal crabs treated with PTU is coincidental.

Factors relating to pigment destruction

Animals injected with 0.5 μC. of tyrosine-C^{14} and maintained for 24 hours on a neutral background with natural lighting were utilized as controls in the following experiments. Animals similarly treated were then transferred to white or black

backgrounds, first for 72 hours and in a subsequent experiment for up to 192 hours. The first experiment indicated that 96% of the label was lost from the melanin of pre-labeled crabs maintained on a white background for 72 hours. The subsequent experiments revealed that the loss of the label from melanin appeared to occur in an exponential fashion for the first 96 hours. Fifty per cent of the label was lost 48 hours after transfer to a white background. Animals maintained on a black background showed no loss of label from melanin after 72 hours. Pre-labeled crabs maintained on a white background for four hours exhibited no measurable destruction of pigment.

The term "albedo" is often used in discussions concerning background responses. It has been classically defined as the ratio of reflected to incident light. The albedo has been postulated as a controlling factor in morphological color changes that occur in fish and in amphibians (Sumner, 1940). For example, animals maintained on a white background will be under conditions of high albedo, whereas those on a black background will be under conditions of low albedo. In the present work, animals on a black background illuminated from below will experience a high albedo. Consequently this treatment is comparable to a white background illuminated from above, from this point of view. Crabs under the above situation compensate for the illumination from below and continue to behave as if they were on a black background illuminated from above. This indicates that an albedo effect (in this simple sense) on pigmentation is probably not the main controlling factor in morphological color changes in the fiddler crab.

In this paper I have tried to elucidate some features of morphological color changes in Crustacea. Early workers in this field worked mainly with fish and amphibians (see review by Sumner, 1940). These workers all seem to accept Babak's hypothesis that factors which promote expansion of the pigment within the chromatophore also promote synthesis of new pigment and formation of new chromatophores. Continued contraction of the pigment within the chromatophores, on the other hand, produces the reverse effect. Those workers making observations on morphological color changes in Crustacea also seem to accept the above hypothesis.

The present work shows that the incorporation of added tyrosine into the black pigment, melanin, occurs independently of the state of pigment expansion or contraction within the chromatophores and, further, that it occurs independently of background shade. The newly formed pigment may be stable over long periods of time.

This discussion assumes that all melanin synthesis occurs within melanophores capable of physiological color changes. If melanin synthesis occurred in other epidermal cells, Babak's hypothesis would not be applicable. Green (1964) has shown that in a related crab, *Ocypode ceratophthalma*, the pigment of newly appearing black chromatophores is capable of dispersal and contraction. However, localization of the precise site of melanin synthesis in the fiddler crab, *Uca pugnax*, will require additional investigation.

If we re-evaluate Babak's hypothesis in terms of the present observations, we must still admit the possibility of a correlation between the state of pigment dispersion and the rate of pigment synthesis. However, if this correlation is to be maintained, the control of synthesis rate must reside in the control of the level of available tyrosine which can enter the synthesis pathway rather than any direct

control over the pathway itself. No information is available concerning the level of free tyrosine in the fiddler crab.

On the other hand, destruction of melanin can be induced by placing the animals on a white background. This occurs despite the fact that the changes induced by this procedure in the state of pigment dispersion are not marked (Brown and Sandeen, 1948; Brown and Hines, 1952). Consequently, the evidence which is available does not support a detailed correspondence between physiological and morphological color changes which would justify invoking the same control system for both phenomena.

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Summary

1. The *Uca* black chromatophore pigment has been identified as a melanin on the basis of: (1) solubility and bleaching reactions; (2) incorporation of tyrosine and dihydroxyphenylalanine into the pigment; and (3) inhibition of tyrosine incorporation by phenylthiourea, a known inhibitor of melanin synthesis.

2. The incorporation of tyrosine-C^{14} into *Uca* melanin is linearly related to micrograms tyrosine injected in the range 0.88–208.00 μg. tyrosine per gram wet weight.

3. Melanin synthesis occurs rapidly under normal conditions and may be related to the influx of free tyrosine.

4. The incorporation of tyrosine-C^{14} into *Uca* melanin is temperature-dependent; the energy of activation, as calculated from the Arrhenius plot of the data, is 5757 calories.

5. Melanin, once labeled, retains the label for considerable periods of time, thereby indicating the stability of the pigment under these conditions.

6. Destalked crabs under similar conditions have a level of tyrosine incorporation approximately 70% higher than do normal animals. This may reflect the increased activity of the epidermis following eye-stalk ablation.

7. Melanin synthesis occurs independently of the state of pigment dispersion, whether naturally-occurring or artificially-induced.

8. Normal animals maintained on a white background under constant illumination show exponential destruction of melanin with a half-time of 48 hours.

9. The available evidence does not support a detailed correspondence between physiological and morphological color changes which would justify invoking the same control systems for both phenomena.

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